Research Article

Analgesic and anti-inflammatory effects of UP1304, a botanical composite containing standardized extracts of Curcuma longa and Morus alba

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ABSTRACT

OBJECTIVE: Though the initial etiologies of arthritis are multifactorial, clinically, patients share the prime complaints of the disease, pain. Here the authors assessed the analgesic and anti-inflammatory effects of UP1304, a composite that contains a standardized blend of extracts from the rhizome of Curcuma longa and the root bark of Morus alba, on rats with carrageenan-induced paw edema.

METHODS: A plant library was screened for bradykinin receptor antagonists. In vivo, the anti-inflammatory and analgesic effects of the standardized composite, UP1304, were evaluated in rats with carrageenan-induced paw edema using oral dose ranges of 100–400 mg/kg. Ibuprofen, at a dose of 200 mg/kg, was used as a reference compound. In vitro, cyclooxygenase (COX) and lipoxygenase (LOX) inhibition assays were performed to evaluate the degree of inflammation.

RESULTS: Statistically significant improvements in pain resistance and paw edema suppression were observed in animals treated with UP1304, when compared to vehicle-treated rats. Results from the highest dose of UP1304 (400 mg/kg) were similar to those achieved by ibuprofen treatment at 200 mg/kg. In vitro, UP1304 showed dose-dependent inhibition of the enzymatic activities of COX and LOX. A half-maximal inhibitory concentration of 9.6 µg/mL for bradykinin B1 inhibition was calculated for the organic extract of C. longa. Curcumin showed Ki values of 2.73 and 58 µg/mL for bradykinin receptors B1 and B2, respectively.

CONCLUSION: Data presented here suggest that UP1304, analgesic and anti-inflammatory agent of botanical origin, acted as a bradykinin receptor B1 and B2 antagonist, and inhibited COX and LOX enzyme activities. This compound should be considered for the management of symptoms associated with arthritis.

Keywords: Curcuma longa; Morus alba; arthritis; chronic pain; bradykinin inhibition


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1 Introduction

Although the initial etiologies of arthritis are multifactorial and dissimilar, clinically, patients present with a prime complaint, pain. Regardless of the initial causes or types of arthritis, during the course of the disease, phospholipids from damaged cell membranes are converted, within joints, to arachidonic acid, creating the initial step in the inflammation cascade that involves the two primary inflammatory enzymes: cyclooxygenase (COX) and lipoxygenase (LOX)\(^\text{[5]}\). The metabolic enzymes COX-1 and COX-2 are involved in the formation of prostaglandins (PGs). PGs are potent hyperalgesic mediators, which modulate multiple sites along the nociceptive pathway, enhancing both peripheral and central sensitizing effects of nociception at the time of inflammation. At the same time, other by-products of arachidonic acid metabolism, mediated by 5'-lipoxygenase (5-LOX), such as leukotrienes (LTs), mainly LT4, have been identified in the synovial fluid of patients with arthritis. These LTs are potent chemotactic factors for neutrophils and are known to be potent stimulating factors for the synthesis of cytokines such as interleukin-1 (IL-1), IL-6 and IL-8 within synovial tissue\(^\text{[23]}\). Therefore, by interrupting the process at the apex, it could be possible to effectively relieve many of the signs and symptoms of inflammation, which are commonly exhibited in patients with arthritis.

More recently, kinins have been implicated in multiple pathophysiological processes, including inflammatory responses, induction of nociception and hyperalgesia\(^\text{[4]}\). There is significant evidence that kinins are rapidly generated after tissue injury and that they seem to modulate most of the events observed during the inflammatory processes, including vasodilatation, increase of vascular permeability, plasma extravasation, cell migration, pain and hyperalgesia\(^\text{[5,6]}\). Most importantly, kinins’ functions are associated with the secondary production of other mediators, including prostanoids, cytokines, mast cell-derived products and nitric oxide (NO), which emphasizes the importance of kinins in the initiation and maintenance of inflammatory and nociceptive processes\(^\text{[7]}\). Kinins exert their biologic effects through the activation of two distinct G-protein-coupled receptors, denoted as bradykinin receptor B1 (BKB1) and bradykinin receptor B2 (BKB2). Bradykinin, blood-derived local-acting kinin, is the most important inflammatory mediator implicated in all 4 principal signs of inflammation, such as swelling, heat, redness and pain. It is generally believed that BKB2 is involved in the acute phase of inflammatory and pain response, whereas BKB1 participates in the chronic phase of the response\(^\text{[6]}\). Hence, inhibiting these peptide hormones could attenuate the main signs and symptoms associated with arthritis.

Currently, non-steroidal anti-inflammatory drugs (NSAIDs) are among the most frequently purchased over the counter solution, as well as the most frequently prescribed substance for use in the management of arthritis pain, where they inhibit the COX-mediated formation of PGs. Nevertheless, this approach which focuses mainly on diminishing pain will only mask the actual etiology, leading to irreversible damage to the joint structure, and rendering the management unsuccessful\(^\text{[8,9]}\).

Through the years, significant progress has been made to counteract symptoms associated with arthritis, by targeting specific pathways involved in disease progression and maintenance. However, the pain management needs of a rapidly increasing population with arthritis are not being met by either conventional pharmaceutical drugs or nutraceuticals. As the goal of treating osteo- or rheumatoid arthritis revolves around minimizing associated joint pain and reducing further inflammation, the search for a botanical alternative that could provide a safe and effective solution to millions who suffer chronic pain from progressive joint degeneration is overdue.

Curcumin, from the rhizomes of *Curcuma longa* Linn (Family: Zingiberaceae), and prenylated flavonoids and stilbenoids, from the root bark of *Morus alba* Linn (Family: Moraceae), possess activities that may be beneficial in the treatment of arthritis. An herbal composite comprised of these well-studied plant extracts, at a specific ratio, may alleviate symptoms associated with arthritis or may slow down progression of the disease.

Here we evaluated the analgesic and anti-inflammatory potential of UP1304, a botanical composite consisting of two standardized extracts from the rhizome of *C. longa* and the root bark of *M. alba*, in carrageenan-induced rat paw edema model. In *vitro*, BKBs, and COX-LOX inhibitions were tested.

2 Materials and methods

2.1 UP1304 preparation

A detailed procedure for the preparation of the composite has been described in US patent # 20150072953\(^\text{[10]}\). The composite contains a proprietary combination of two standardized ethanol extracts from root barks of *M. alba* and rhizomes of *C. longa*, with not less than 10% curcumin and 2% mulberroside A in the final blend.

2.2 BKB-binding assay

Upon confirmation of the lead extract of *C. longa* and its active constituents, pure curcumin compound, purchased from Sigma (Sigma, C1386-50G), was tested for its specificity in BKB1- and BKB2-binding assays at
concentrations ranging from 200 μmol/L to 5 nmol/L.

2.2.1 BKB1 radioligand-binding assay

The BKB1 radioligand-binding assay was conducted to determine the BKB1 receptor inhibition activity of plant extracts. A total of 6397 organic extracts (OE, 3920) and aqueous extracts (AE, 2477) from Unigen’s library were screened for their ability to inhibit the BKB1 receptor at a concentration of 166 μg/mL. Half-maximal inhibitory concentration (IC50) values were determined using the same method, with serial dilutions at concentrations ranging from 400 μg/mL to 5 ng/mL, in order to obtain a dose-response curve. The lead OE extract was then further fractionated and subjected to a high-through-put assay for BKB1-binding activity. In brief, this method used human IMR-90 lung fibroblast membranes (Sigma-Aldrich, St. Louis, MO, USA), stimulated with 0.1 μg/mL IL-1β (Sigma-Aldrich, St. Louis, MO, USA) in a modified hydroxyethylpiperazine ethanesulfonic acid (HEPES) buffer (pH=7.4); these membranes were incubated with OE extracts at 166 μg/mL in the presence of 0.9 nmol/L [3H](Des-Arg9)-kallidin, for 60 min, at room temperature. After incubation, the membranes were filtered and washed five times with modified Dulbecco’s phosphate-buffered saline buffer (pH 7.4). BKB1 membrane-bound cell numbers were determined.

2.2.2 BKB2-binding assay

Inhibition of BKB2-binding activity of curcumin was examined with methods similar to those described for the BKB1 with some modifications. Bradykinin radioligand-binding assay was carried out using an assay buffer composed of 24 mmol/L 2-[[1,3-dihydroxy-2-(hydroxymethyl)propan-2-yl]amino]ethanesulfonic acid (TES, pH 6.8), 1 mmol/L 1,10-phenanthroline and 0.3% bovine serum albumin (BSA). BKB2 used in the assay was from CHO-K1 (Chinese hamster ovary) cells expressing recombinant human BKB2. The ligand was [3H]-bradykinin at 0.2 nmol/L. The reaction mixture was incubated for 90 min at room temperature and BKB2 membrane-bound cell numbers were counted.

The binding curve was plotted from a non-linear regression fit (using GraphPad Prizm software) and the concentration required to produce IC50 of the enzymatic reaction, also known as the inhibitory constant value, or Ki value was computed manually using the Cheng-Prusoff algorithm. The potency of a test material is inversely correlated with its Ki value (i.e., the lower the Ki, the more potent the material).

2.3 COX-LOX enzyme inhibition assay

2.3.1 COX inhibition

The COX enzyme inhibition effect was evaluated with a commercial colorimetric COX (ovine) inhibitor screening assay kit (Cayman Chem., Co., Cat # 760111). In brief, 150 μL of assay buffer, 10 μL of heme, 10 μL of COX-1 or COX-2 enzyme and 20 μL of UP1304, at a concentration of 10, 25, 50 or 100 μg/mL, were added to a 96-well plate. The plate was shaken carefully for a few seconds and incubated at 25 °C for 5 min. Colorimetric substrate solution (20 μL) and arachidonic acid were added to initiate the reaction. After shaking carefully, it was incubated for 10 min at 25 °C and the absorbance of each well was measured at 590 nm using a plate reader (VICTOR™ X3 PerkinElmer, Waltham, MA, USA).

2.3.2 5-LOX inhibition activity

Similarly, the 5-LOX inhibition activity was tested by commercial 5-LOX inhibitor screening assay kit (Cayman Chem., Co., Cat # 760700). In brief, 90 μL of 5-LOX enzyme and 10 μL of UP1304, at concentrations of 10, 25 or 50 μg/mL were combined in a 96-well plate; the plate was shaken carefully for a few seconds. Substrate (linoleic acid 10 μL) was added to each well of the plate in order to initiate the reaction and the plate was placed on a shaker for 5 min. Chromogen (100 μL) was then added to each well to stop enzyme catalysis. The 96-well plate was placed on a shaker for 5 min and the absorbance of each triplicate well was measured at 490 nm using a plate reader.

2.4 Animals

Lewis rats (n=75), purchased from Charles River Laboratories (Wilmington, MA, USA), at the age of 8 weeks, were acclimated upon arrival for a week, before being assigned randomly to their respective groups. Rats were housed in polypropylene cages (3 per cage) and individually identified by numbers on their tails. Each cage was covered with a wire bar lid and a filtered top. Individual cages were identified with a card indicating the project number, test article, dose level, group and animal numbers. Harlan T7087 soft cob beddings was used and changed at least twice weekly. Animals were provided with fresh water and rodent chow diet # T2018 (Harlan Teklad, 370W, Kent, WA, USA) ad libitum and were housed in a temperature-controlled room (22.2 °C) on a 12 hour light-dark cycle. All animal experiments were conducted according to institutional guidelines congruent with guidelines for the care and use of laboratory animals with approval reference number UAS-CM1304.

2.5 Carrageenan-induced rat paw edema

Rats were sedated with 2.5% isoflurane (Piramal Healthcare; lot: A19E14A), and inflammation and pain sensitivity were induced by intraplantar injection of 100 μL of 1% [w/v] carrageenan θ (Sigma, St. Louis, MO, USA; lot # 1408463V) into the plantar surface of the right hind paw of each rat, marking time 0 (T=0)[13,14]. Rats were acclimated in a procedure room for 20–30 min before each measurement was taken.
2.6 Effects of UP1304 on paw edema

One hour after carrageenan inoculation, animals (n=5 per group) were orally gavaged with ibuprofen at 200 mg/kg (Spectrum Chemical MFG, Gardena, USA; lot # ZG0097), as a positive control; 0.5% carboxymethyl cellulose (CMC), as the vehicle control, or UP1304 at doses of 100, 200, 300 or 400 mg/kg. The dose-correlated efficacy and comparison among individual components were assessed at T0 (before induction), and 1, 2, 4 and 6 h after induction. The merit of combining C. longa and M. alba was also evaluated in this model, using Colby's equation\(^{[14]}\). In this method, a formulation with two or more materials is presumed to have unexpected synergy if the observed value of a certain endpoint measurement is greater than the hypothetically calculated values\(^{[15]}\). Paw edema and pain sensitivity percent change values of C. longa (150 mg/kg) and M. alba (150 mg/kg) at 1, 3 and 5 h after treatment were used to determine the calculated efficacy values and compared to the observed percent change values of the composite UP1304 (300 mg/kg) at the specified time points.

2.6.1 Allodynia evaluation

Allodynia was evaluated by measuring responsiveness to a tip of Randell-Salitto (IITC, Woodland Hills, CA, USA; model # 2888) applied perpendicularly to the central plantar surface of the right hind paw. A positive response to the applied pressure, noted by sharp withdrawal of the paw, was recorded automatically by an electronic von Frey anesthesiometer (2 390 series Electrovonfrey, IITC, Woodland Hills, CA, USA)\(^{[16]}\). Mechanical allodynia was evaluated before carrageenan inoculation, and thereafter 1, 2, 4 and 6 h.

2.6.2 Paw edema test

Paw edema was measured with the use of a plethysmometer (IITC, Woodland Hills, CA, USA; model 520) at time 0 (before carrageenan injection), and 1, 2, 4 and 6 h after carrageenan injection\(^{[13,14,16]}\).

2.7 Statistical analysis

Data were analyzed using SigmaPlot (Version 11.0, Systat Software, Inc., San Jose, CA, USA). The data are represented as mean ± standard deviation. Statistical significance among groups was calculated by means of single-factor analysis of variance (ANOVA) and by Student’s t-test. P values less or equal to 0.05 (P ≤ 0.05) were considered significant. When normality test failed, for non-parametric analysis, the Mann-Whitney test and Kruskal-Wallis test were used.

3 Results

3.1 Receptor-binding assays

The organic extract of C. longa showed strong receptor inhibition activity when screened at a concentration of 166 µg/mL. The extract inhibited BKB1 binding and had an IC\(_{50}\) of 9.6 µg/mL. Curcuminoids were determined to be the compounds most associated with the activity (data not shown). Our data indicated that curcumin had greater affinity to BKB1, with much stronger inhibition activity (Ki value = 2.173 µg/mL), than to BKB2 (Ki value = 58 µg/mL); hence we considered it to be a selective BKB1 antagonist. Aqueous extracts had very little enzyme inhibition activity, and were excluded from further studies.

3.2 COX and LOX inhibitions

As shown in Table 1, UP1340 inhibited COX-1 and COX-2 enzymes in a dose-dependent manner, with IC\(_{50}\) of 40.5 and 74.1 µg/mL respectively. Similarly, an IC\(_{50}\) of 26.3 µg/mL was calculated for the 5-LOX enzyme.

3.3 Carrageenan-induced edema

3.3.1 Composite UP1304 and extracts of C. longa and M. alba

Analgesic and anti-inflammatory activities of UP1304, at a dose of 300 mg/kg, were tested in rats with carrageenan-induced paw edema. As seen in Table 2, UP1304 was more effective at inhibiting pain sensitivity (P values: vs M. alba, 0.04, 0.001 and 0.01; vs C. longa 0.000 1, 0.03 and 0.03) and paw edema (vs M. alba, 0.05, 0.15 and 0.05; vs C. longa, 0.003, 0.04 and 0.02) than either C. longa or M. alba given alone, after 1, 3 and 5 h. Compared with the positive control, ibuprofen (200 mg/kg), UP1340 was less effective at reducing paw edema (P values of 0.03 and 0.05) after 3 and 5 h of treatment and pain sensitivity.

Table 1 Effects of different concentrations of UP1304 on COX-1/-2 and 5-LOX enzyme activity

<table>
<thead>
<tr>
<th>Dose (µg/mL)</th>
<th>COX-1 (%)</th>
<th>COX-2 (%)</th>
<th>5-LOX (%)</th>
<th>COX-1/COX-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>UP1304 10</td>
<td>17.3±0.23</td>
<td>14.3±1.11</td>
<td>25.7±2.59</td>
<td>1.21</td>
</tr>
<tr>
<td>UP1304 25</td>
<td>39.8±0.35</td>
<td>19.7±1.21</td>
<td>56.3±1.04</td>
<td>2.02</td>
</tr>
<tr>
<td>UP1304 50</td>
<td>57.6±0.06</td>
<td>35.8±1.29</td>
<td>75.3±0.40</td>
<td>1.61</td>
</tr>
<tr>
<td>UP1304 100</td>
<td>107.8±0.61</td>
<td>63.6±0.49</td>
<td>-</td>
<td>1.69</td>
</tr>
</tbody>
</table>

Data of inhibitory rates are represented as mean ± standard deviation. COX: cyclooxygenase; 5-LOX: 5′-lipoxygenase.
3.3.2 Synergistic effect of Curcuma and Morus extracts
Carrageenan-induced paw edema was utilized to evaluate a possible synergy of extracts from Curcuma and Morus combined in a 1:1 ratio, using Colby’s method. When rats were given UP1304 at a dose of 300 mg/kg, the observed reductions in inflammation and pain sensitivity were greater than the theoretically calculated values at all experimental time points (1, 3 or 5 h after treatment; Table 3). These findings suggest that formulation of two standardized extracts from Morus and Curcuma at a 1:1 ratio has a greater benefit than using either Morus or Curcuma extract alone.

3.3.3 Dose-correlated activity of UP1304
As shown above, UP1304 showed superior anti-inflammatory and analgesic activities to individual extracts of C. longa or M. alba, at a dose of 300 mg/kg. To determine the optimum dosage of UP1304, doses of 400, 300, 200 and 100 mg/kg were administered orally to rats 1 h after induction of carrageenan-induced paw edema. As seen in Figures 1 and 2, there was a clear dose-correlated, statistically significant inhibition in hypersensitivity and inflammation after UP1304 treatment, relative to the vehicle control. As expected, the positive control ibuprofen showed statistically significant inhibition of both pain sensitivity and paw edema at each time point considered (P≤0.05). When compared with ibuprofen, UP1304 at the highest dose (400 mg/kg) showed similar efficacy (P values of 0.48 and 0.86 in pain sensitivity; 0.86 and 0.26 in paw edema inhibitions) after 1 and 5 h. However, 3 h after treatment, the inhibitory effects of ibuprofen in pain sensitivity were greater than those of the extracts tested (P=0.01), and there was no significant differences in paw edema (P=0.06). After treatment with UP1304 or ibuprofen at the same dose (200 mg/kg), statistically significant differences were observed in both pain sensitivity (P≤0.05) and paw edema (P≤0.05) at each time point, except paw edema at 1 h after treatment (P=0.12).

Table 2 Analgesic and anti-inflammatory activity in carrageenan-induced rat paw edema model

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose (mg/kg)</th>
<th>n</th>
<th>Paw edema</th>
<th>Pain sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>1 h</td>
<td>3 h</td>
</tr>
<tr>
<td>Morus extract</td>
<td>300</td>
<td>5</td>
<td>45.1±0.1</td>
<td>38.3±0.3</td>
</tr>
<tr>
<td>Curcuma extract</td>
<td>300</td>
<td>5</td>
<td>41.8±0.1</td>
<td>35.3±0.2</td>
</tr>
<tr>
<td>Ibuprofen</td>
<td>200</td>
<td>5</td>
<td>53.9±0.6</td>
<td>59.0±0.4</td>
</tr>
<tr>
<td>UP1304</td>
<td>300</td>
<td>5</td>
<td>51.4±0.1</td>
<td>△</td>
</tr>
</tbody>
</table>

Hypersensitivity threshold and paw edema were determined by subtracting 2, 4 and 6 h individual values from their respective T0 value. Data are expressed as mean of percent reduction relative to vehicle with standard deviation and reported as 1, 3 and 5 h after treatment. △P≤0.05, △△P≤0.01, vs Morus extract group; ▲P≤0.05, ▲▲P≤0.01, vs Curcuma extract group; □P≤0.05, □□P≤0.01, vs ibuprofen group.

Table 3 Unexpectedly enhanced analgesic and anti-inflammatory activity of UP1304 in carrageenan-induced rat paw edema model

<table>
<thead>
<tr>
<th>Composition</th>
<th>Compound</th>
<th>Dose (mg/kg)</th>
<th>n</th>
<th>Paw edema</th>
<th>Pain sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1 h</td>
<td>3 h</td>
</tr>
<tr>
<td>Extract</td>
<td>Curcuma</td>
<td>150</td>
<td>5</td>
<td>27.7±0.2</td>
<td>24.3±0.1</td>
</tr>
<tr>
<td></td>
<td>Morus</td>
<td>150</td>
<td>5</td>
<td>31.4±0.1</td>
<td>26.2±0.1</td>
</tr>
<tr>
<td>UP1304</td>
<td>Expected*</td>
<td>–</td>
<td>–</td>
<td>50.4±0.2</td>
<td>44.1±0.2</td>
</tr>
<tr>
<td>Observed†</td>
<td>300</td>
<td>5</td>
<td>51.4±0.1</td>
<td>46.6±0.4</td>
<td>41.4±0.3</td>
</tr>
</tbody>
</table>

*Expected: Calculated value according to Colby’s method = A – B. A= Percent change value of Curcuma at extract 150 mg/kg + Percent change value of Morus extract at 150 mg/kg; B= Percent change value of UP1304 at 300 mg/kg. †Observed: Data observed when rats was orally administered with UP1304 at 300 mg/kg.
The major bioactive components of the turmeric plant, curcumin, as well as the prenylated flavonoids and stilbenoids from the root bark of *M. alba*, possess activities that may provide benefits in the management of chronic pain and arthritis. For instance, curcumin has been shown to have potent anti-inflammatory and anti-catabolic effects through the NF-κB pathway\[18,19\] and transcription activator protein-1 (AP-1)\[20\]; these include reducing the IL-1β-mediated up-regulation of NF-κB targets, such as matrix metalloproteinase-1 (MMP-1), MMP-3, MMP-9, and COX-2, as well as reducing chondrocyte apoptosis. Curcumin also decreased the IL-1β-stimulated production of NO and PGE\(_2\) in chondrocytes, through signal transduction pathways involving p38 MAPK, JNK, NF-κB and AP-1\[21\]. Curcumin has also inhibited IL-1β-stimulated proinflammatory mediators, NO, PGE\(_2\), IL-6, IL-8, and MMP-3 production in human articular chondrocytes\[22\].

Similarly, a variety of bioactive compounds from *M. alba* root bark have showed *in vivo* or *in vitro* anti-inflammatory activity. For example, oxyresveratrol inhibited NO production, reduced inducible NO synthase (iNOS) expression, inhibited PGE\(_2\) production and suppressed the activation of NF-κB\[23\]. Total flavonoids from the root bark\[24\] and prenylated flavonoids\[25\] from *M. alba* extract were reported to have the potential to inhibit both NO production and iNOS, as well as to reduce pro-inflammatory mediators such as COX-2, IL-1β and IL-6. These collective pharmacological activities of *C. longa* and *M. alba* suggest suitability for use in treatment of arthritis. In light of the well-established potential activities of these plant materials, we hypothesized that enhanced anti-inflammatory and analgesic efficacy may be achieved by a combination of standardized extracts of *C. longa* and *M. alba*. By combining these extracts in a 1:1 ratio, we produced a composite that we called UP1304. It was also hypothesized that UP1304 would possess all of the documented qualities contributed by the individual herbal components, recommending its use for treatment of arthritis. We tested these hypotheses using an established animal model.

Findings in the present study seemed to support the above hypotheses by demonstrating efficacy of UP1304 in multiple experiments\[26\]. The current report depicts data from the carrageenan model only. To the best of our knowledge this is the first time either of the components of the composite have been shown to inhibit BKB, possibly associated with pain. Bradykinin is an important and gastrointestinal side effects associated with long term use of selective or non-selective NSAIDs reinforce the need to develop botanical alternatives with anti-inflammatory and analgesic activities without the side effects that accompany NSAIDs.

Figure 1 Dose-correlated anti-inflammatory activity of UP1304
Rats (n=5) were gavaged with composite UP1304, ibuprofen and vehicle 1 h after carrageenan-induced paw edema. Paw edema data as a measure of inflammation are expressed as mean ± standard deviation. *P ≤ 0.05, **P ≤ 0.01, vs vehicle; ***P≤0.05, vs ibuprofen group.*

Figure 2 Dose-correlated analgesic activity of UP1304
Rats (n=5) were gavaged with composite UP1304, ibuprofen or vehicle 1 h after carrageenan-induced paw edema. Pain sensitivity data are expressed as mean ± standard deviation. *P ≤ 0.05, **P ≤ 0.01, vs vehicle; ***P≤0.05, vs ibuprofen group.*

**4 Discussion**

Pain, one of the cardinal signs of inflammation, is the most common clinical manifestation of arthritis. Based on mounting evidence on changes in joints of both arthritic patients and animal models, it is recognized that a movement of inflammatory cells into the synovium and synovial fluid, leads to hyperplasia and activation of resident synovial and inflammatory cells, forming the basis of the pathogenesis of the disease\[17\]. Despite the extraordinary advances in therapeutic discovery, there still is a gap in the development of a safe, effective and economical therapy for managing chronic inflammatory pain in arthritis. In particular, the adverse cardiovascular
inflammatory mediator involved in edema formation and serves as a trigger for inflammatory pain. Evidence suggests that the pathophysiological process of tissue damage and inflammation are closely associated, with bradykinin as a primary target in producing and sustaining pain and hyperalgesia\textsuperscript{[27–29]}. Among the most frequently described mechanisms of bradykinin, the associations to hyperalgesia are (a) the direct activation of nociceptors, (b) sensitization of nociceptors through the production of prostanoids, such as PGE\textsubscript{2}, or (c) the release of other mediators relevant to pain and hyperalgesia\textsuperscript{[27]}. While pain and swelling from acute tissue damage are believed to be mediated by the BK\textsubscript{B2}, it has recently been suggested that in chronic inflammation, BK\textsubscript{B1} plays an important role in the maintenance of hyperalgesia, which is considered an adaptive mechanism that occurs peripherally and centrally, following the sustained activation of nociceptors. Taking these facts into consideration, multiple specific antagonists for BK\textsubscript{B1} or BK\textsubscript{B2} have been assessed for their analgesic and anti-inflammatory activities in acute and chronic inflammatory pain\textsuperscript{[28,29]}. In the present study the UP1304 composite showed strong inhibition in pain sensitivity and inflammation in all models. These reductions in pain sensitivity and/or anti-inflammatory activities could partially be explained by the strong BK\textsubscript{B} inhibition affinity of curcuminoids in UP1304.

Carrageenan injection into the intraplantar region of rat hind paw produces a classic model of hyperalgesia and edema. The hyperalgesia exhibited by the model is an essential feature of inflammatory pain, which consists of the action of the COX/LOX-mediated increase in prostaglandins and leukotrienes, leading to peripherally and centrally mediated sensitization,\textsuperscript{[30]} accompanied by increased tissue fluid and plasma protein exudation, and forming a localized edema at the site of injection\textsuperscript{[13]}. In our study, considerable reduction in pain sensitivity and paw edema were observed in rats given UP1304 orally. These inhibitions in the carrageenan-induced rat paw edema model could be due to the association of bradykinin with other proinflammatory cytokines. In support of our hypothesis, previous studies have shown a link between bradykinin and the production of TNF-\(\alpha\), which initiated a cascade of cytokine release, including IL-1, IL-6 and IL-8 in the carrageenan-induced mechanical hyperalgesia model\textsuperscript{[31]}. Activation of IL-1 and IL-6, can stimulate the release of hyperalgesic COX products, such as PGE\textsubscript{2}, causing inflammatory hyperalgesia. In the inflammation process of both the carrageenan-induced paw edema model, and the low-dose lipopolysaccharides (LPS)-induced models, bradykinin acting upon BK\textsubscript{B2} triggered the release of TNF-\(\alpha\); however, treatment with the BK\textsubscript{B2} antagonist, HOE140, at a dose of 1 mg/kg, showed inhibition of this hyperalgesic response\textsuperscript{[32]}. Unfortunately, HOE140 failed to inhibit pain sensitivity caused by high doses of LPS, suggesting the presence of another mediation in addition to BK\textsubscript{B2}. Subsequently, multiple studies were carried out to characterize the role of BK\textsubscript{B1} in mediating the hyperalgesic response to persistent inflammatory stimuli as an apex in association with TNF-\(\alpha\)-driven cascade of cytokine release followed by induction of COX-mediated prostanoid productions\textsuperscript{[33]}. These previously documented findings suggest a possible mechanism of action for UP1304 in alleviating pain and inflammation: through the inhibitions of BK\textsubscript{B1} and hence disruption of the proinflammatory cascades. In fact, major enzymes COX/LOX known to be involved in inflammatory process have been moderated by UP1304 in vitro. Interestingly, in the present study we found an unexpected synergy in the combination of Curcuma extract with Morus extract. The beneficial effects of UP1304 treatment exceeded the sum of the effects of its constituents. Complementing our findings, previously curcumin has shown the ability to affect arachidonic acid metabolism by blocking the phosphorylation of cytosolic phospholipase A\(_2\), decreasing the expression of COX-2 and inhibiting the catalytic activities of 5-LOX\textsuperscript{[34]}.

We have documented statistically significant improvement in pain sensitivity and suppression of paw edema in animals orally treated with UP1304, compared to vehicle-treated rats of the same model\textsuperscript{[35–37]}. Also supporting these findings, oxyresveratrol and mulberroside A, from the root bark of M. alba, have been reported to have an anti-inflammatory effect on the carrageenan-induced paw edema model in rats at a dosage of 7.5 mg/kg and 50 mg/kg respectively\textsuperscript{[32]}. Similarly, another report showed inhibition of PGE\textsubscript{2} and suppression of COX-2 mRNA in both carrageenan-induced paw edema and peritonitis in mice treated with Morus extract\textsuperscript{[35]}. In a similar study, curcumin was administered orally for two consecutive weeks, at a dose of 50 mg/kg, to rats that were subjected to carrageenan-induced inflammation. After the last dose, statistically significant suppression of inflammatory paw edema (69\%) and tissue TNF-\(\alpha\) (32\%) were observed\textsuperscript{[36]}. In another study, where rats were provided with dietary curcumin at 0.2\% daily for 10 weeks, a 12\% improvement in paw edema and a significant decrease in 5-LOX activity in the polymorphonuclear lymphocytes were observed in the carrageenan-induced rat paw edema model\textsuperscript{[37]}.  

5 Conclusion

In the present study, UP1304, a composite of standardized extracts from the rhizome of C. longa and the root bark of M. alba, inhibited the activity of bradykinin, COX and LOX. It also reduced pain and inflammation
associated with the carrageenan-induced rat paw edema model. The clinical use of UP1304 could be rationalized by the fact that the primary biomarkers frequently isolated with patients experiencing chronic arthritis were modulated by UP1304 and its constituents. Therefore, UP1304 could potentially be considered as a dietary supplement product for the management of arthritis.

6 Author contributions

MY and YCL conceived and designed the study, carried out study, data calculation, statistical analysis and data interpretation, and drafted/editied the manuscript. BM assisted in conducting the in vivo studies. PJ, MH, JBN, and MRK conducted structure elucidations, identification, material sourcing and extractions. QI, EJH, LB and MC conceived the study, participated in its design, interpreted data, and edited the manuscript. All authors read and approved the final manuscript.

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9 Competing interests

All authors are currently Unigen employees and therefore, have competing financial interests.

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